

**CLINICAL,
GENETICAL AND BIOCHEMICAL
ASPECTS OF FACTOR VIII**

by

Lars Holmberg

Malmö 1974

**From the Coagulation Laboratory and the Department of
Paediatrics, University of Lund, Allmänna
Sjukhuset, Malmö, Sweden**

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The survey is based on the following papers:

1. Holmberg, L. and Nilsson, I.M.: Immunologic studies in haemophilia A. Scand. J. Haemat. 10:12-16, 1973.
2. Holmberg, L. and Nilsson, I.M.: Genetic variants of von Willebrand's disease. Brit. med. J. 3:317-320, 1972.
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HISTORICAL REMARKS AND INTRODUCTION

Coagulation factor VIII or the antihaemophilic factor A (AHF, AHG) is defined as the factor missing in the commonest form of classical haemophilia, haemophilia A. The only unequivocal evidence that a substance is identical with factor VIII is thus that it can correct, in vitro and vivo, the coagulation defect in haemophilia A.

The clinical picture and the sex-linked inheritance of classical haemophilia have long been known. John C. Otto, an American physician, gave the first significant description of haemophilia in 1803. In Europe it was first described by Nasse (1820). The name of the disease was presumably coined by Schönlein in the 1820s (Brinkhous 1965). The early literature on haemophilia has been reviewed by many, such as Schloessman (1930), Brinkhous (1965) and Quick (1966). In the last two decades Ikkala (1960), Sjølin (1960), Ramgren (1962) and Ahlberg (1965) of the Nordic countries have published investigations of historical, clinical, genetical and other aspects of the disease.

Investigation of factor VIII must always be intimately connected with the study of haemophilia. Wright (1893) was the first to emphasise a prolonged clotting time as a characteristic of haemophilia. In 1905 Sahli demonstrated that the amount of fibrinogen in haemophilia is normal. The cause of the disease was thought to be some abnormality of the platelets, but in 1911 Addis prepared from normal plasma a globulin fraction that could normalise the coagulation time of haemophilic plasma in vitro, while a corresponding fraction from haemophilic plasma could not. Patek and Stetson (1936) and Patek and Taylor (1937) showed that normal platelet-free plasma or a "globulin substance" prepared from normal plasma could normalise the prolonged clotting time not only in vitro, but also in vivo. The substance was later called antihaemophilic globulin (Lewis et al 1946), since it had been shown that it was not identical with any of the classical coagulation factors, especially not with prothrombin (Howell and Cekkada 1926). The consumption of prothrombin during clotting, however, was seriously impaired in haemophilia plasma but could be increased to normal by addition of small amounts of tissue thromboplastin

(Brinkhous 1939). These observations strengthened the theory of a deficient formation of plasma thromboplastin. Both platelets and plasma factors were needed to generate this principle, but in haemophilia the defect was in the latter (Quick 1947, Brinkhous 1947).

It was also realised that haemophilia is not a uniform disease. The works of Pavlovsky (1947) and of others revealed the existence of another haemophilia factor besides AHC. Two forms of haemophilia could be distinguished, viz. haemophilia A with lack of the anti-haemophilic globulin (AHG, AHF, factor VIII) and haemophilia B with lack of B-factor (factor IX). Despite wide differences in the properties of these two factors, a given degree of deficiency of one of them produces the same clinical picture as a corresponding deficiency of the other (Koller 1954, Famgren 1962). In most parts of the world haemophilia A is four times as common as haemophilia B (see Farngren 1962).

The 1950s witnessed the development of methods permitting more precise quantitation of factor VIII activity in plasma. The ability of a patient's plasma to correct the defect of haemophilia A plasma was compared with that of normal standard plasma. Such a test was described by Langdell et al (1953) and was based on the partial thromboplastin time. Various modifications of the procedure were worked out (Nilsson et al 1957a, Waaler 1959, Egeberg 1961). Hardisty and Macpherson (1962) and Veltkamp et al (1968) added a kaolin suspension to standardise contact activation. Another method for quantifying factor VIII was based on the thromboplastin generation test (Pitney 1956, Biggs 1957).

The advent of the more sensitive methods for determining factor VIII made it possible to shed light on another haemorrhagic disorder. In 1953 Alexander and Goldstein described two cases with a combined defect of haemostasis, namely a prolonged bleeding time and decreased factor VIII activity. Similar cases were afterwards observed by other authors (see M. Blombäck 1958). Schulman et al (1955) called the disease vascular haemophilia, while Singer and Ramot (1956) used the term pseudohaemophilia B.

Nilsson et al (1957b) showed that the haemorrhagic disease occurring on the Åland islands and described by von Willebrand in 1926, was characterised not only by a prolonged bleeding time, but also by abnormally low factor VIII activity. This finding clearly showed that the bleeding disease observed in various quarters of the world was identical with that described by von Willebrand. The

disease is believed to have an autosomal and dominant mode of inheritance (von Willebrand and Jürgens 1933, Nilsson et al 1957a, Barrow and Graham 1964).

It was gradually realised that changes in factor VIII activity could occur in situations other than congenital bleeding diseases. Stress, such as strenuous exercise, and infusion of adrenaline enhances the factor VIII activity (Rizza 1961, Ingram 1961, Ikkala et al 1963) in normal persons. In several diseases a rise in factor VIII activity has also been shown to occur (Zetterquist and v. Francken 1963, Egeberg 1962a), but it could sometimes be decreased (Niléhn 1962).

Biochemical characterisation of factor VIII protein has widened our knowledge of diseases with deviations of factor VIII activity. But the characterisation is far from complete, largely because of the lability of the factor and its low concentration in the plasma. The early endeavours to obtain factor VIII concentrates have been reviewed by M. Blombäck (1958). Since then, and especially during the last few years, methods for the extensive purification of factor VIII have become available. The present investigation was begun with the aim to examine with new techniques congenital and acquired diseases with aberrations in factor VIII activity and, if possible, to elucidate their biochemical basis.

METHODS

Collection of blood. Blood was collected with the silicone technique and citrated platelet-poor plasma (PPP) was prepared as described by Nilsson et al (1957a). Platelet-rich plasma (PRP) was prepared from venous blood, which was anticoagulated with 0.077 M EDTA solution in proportions 7.5 ml EDTA solution per 92.5 ml blood. The blood was centrifuged at 20°C and 185 g for 10 min. Washed platelets were prepared from PRP, which was recentrifuged at 2,400 g for 30 min, washed twice in Tris buffered EDTA (1 % Na₂EDTA in Tris buffered saline, pH 7.3) and twice in Tris buffered saline (1 part 0.25 M Tris-HCl buffer, pH 7.3 and 2 parts of 0.85 % NaCl).

Cryoprecipitate for Sepharose 6 B chromatography was prepared according to the principle of Pool and Shannon (1965). 400 ml plasma from each normal person or patient was obtained with the double plasmapheresis technique (Fenwal PC-210). Samples of 200 ml citrated plasma were transferred into 600 ml plastic bags (Fenwal TA-1) and frozen in CO₂-methanol as flat sheets; they were thawed for 12-14 hours at +4°C and centrifuged at the same temperature.

Coagulation time, recalcification time, prothrombin consumption, one-stage prothrombin time, P&P, factors V and IX were done according to methods described by Nilsson et al (1961) and factors XI and XII according to Karaca and Nilsson (1972). Fibrinogen was assessed according to Nilsson and Olow (1962a) and with an immunochemical method according to Karaca et al (1971). Fibrinolytic activity was measured according to Nilsson and Olow (1962b) and fibrinogen degradation products with Niléhn's method (1967).

Bleeding time was examined with Duke's method and with Ivy's method as modified by Borchgrevink and Waaler (1958). Normal values are 1-5 respectively 6-12 min (Nilsson et al 1963).

Platelet count (Björkman 1959). Normal range 155,000-365,000/cu.mm.

Platelet adhesiveness. The method of Salzman (1963) (normal value >20 %) and the method of Bowie et al (1969) (normal value >70 %).

Platelet aggregation studies with Born's aggregometer according to Cronberg et al (1970).

Clot retraction, platelet factor 3, platelet factor 3 availability, ADP release from PRP with thrombin and from washed platelets with kaolin according to methods detailed by Karaca and Nilsson (1972).

Aggregation with Ristocetin was performed 1) according to Howard

and Firkin (1971) on PRP with different final concentrations (1.0, 1.2, 1.5 mg/ml) of Ristocetin; 2) quantitatively according to Weiss et al (1973a) on washed platelets. Values with the latter method in 10 normal controls were 60-120 %. Ristocetin was kindly supplied by H. Lundbeck & Co. A/S, Copenhagen, Denmark.

Factor VIII activity was assayed on platelet-rich haemophilia A plasma (factor VIII activity $<1\%$) in a recalcification system (Nilsson et al 1957a, Nilsson et al 1959). The haemophilia A test substrate was obtained with the silicone technique and centrifuged at 185 g for 20 min. It was checked that the haemophilic plasma contained at least 200,000 platelets/cu.mm. The plasma was stored at -60°C for at most one month before use. The plasma to be tested was prepared by immediate centrifugation of the citrated blood for 20 min at $2,400\text{ g}$ at room temperature and was stored at -60°C until examined. The 100 % standard plasma was a mixture of plasma from 20 normal donors. The plasma to be tested was assayed at dilutions of 1:50, 1:100 and 1:200 in 0.9 % saline. We have had more than 15 years' experience with this method at our laboratory. It has been shown that the method is very reliable in the hands of experienced technicians. Unlike several other methods the reproducibility of the values obtained with both plasma and factor VIII concentrates is very good. This has been shown by comparison with international standards (Miller-Andersson 1974). The normal range is 60-160 % with a standard deviation of 17.5 % (Nilsson et al 1957a). The error of the method at different levels of factor VIII activity has been calculated by Nilsson (1974).

Purification of factor VIII was done with the method of van Mourik and Mochtar (1970), as described in Paper 9.

Agarose gel chromatography of plasma on Sepharose 2 B and 6 B was performed on $1.5 \times 30\text{ cm}$ siliconised glass columns. The columns were eluted at $+4^{\circ}\text{C}$ with an imidazole buffer, pH 7.3 at a rate of 9 ml/hour.

Preparation of heterologous antiserum to factor VIII. 1 ml of purified factor VIII solution was emulsified with Freund's complete adjuvant and injected into the foot pads and the neck skin of rabbits. The same amount was given as a booster dose 3 weeks later, and the rabbits were bled after a further 2 weeks. The blood was allowed to clot for at least 2 hours and the serum was separated off. On immunodiffusion and crossed immunoelectrophoresis the antiserum generally produced only one line with plasma and was then not absor-

bed. For neutralisation experiments the antiserum was heated at 60°C for 1 hour and adsorbed with BaSO₄.

Quantitative determination of factor VIII antigen was performed with Laurell's electroimmuno assay (1972). In the beginning determinations were made on cryoprecipitate, but later after a modification of the method plasma was used as a source of antigen. The concentration of antibody in the gel was such that the precipitate with undiluted plasma had a height of about 30 mm. Plasma was tested undiluted and in dilutions 1:2 and 1:4. The concentration of agarose was 1 %. The electrophoresis was run for 16 hours at 5 volts/cm, which gave better outlined precipitates than a higher voltage for a shorter time. 5 µl in the wells was better than 10 µl. The reproducibility of the method was good. In double determinations the second value never differed from the first by more than 10 % of that value. The range of values in 32 normal persons of both sexes was 47-194 %, mean 94.0, S.D. 36.1. Median 85. Normal values were set 50-175 %. Of the 32 values two fell below (47 and 48 % respectively) and one above this range.

Crossed immunoelectrophoresis in agarose was done according to Garrot (1972). Double diffusion in agarose according to Ouchterlony (1967).

Ability of factor VIII to neutralise a human inhibitor was assayed according to a modification of the method of Denson et al (1969), described in Paper 5.

FITC (fluorescein isothiocyanate)-conjugation of rabbit antiserum to factor VIII was performed according to Holborow and Johnson (1967), and the conjugation mixture was chromatographed on DEAE-cellulose with stepwise elution (Paper 6).

SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969). The concentration of acrylamide in the gels was 2.7 % or 5 %.

Iso-electric focusing in polyacrylamide gel was done with a modification of the method of Jeppsson and Berglund (1972), described in Paper 9.

Reduction of factor VIII protein was done in 6 M guanidine hydrochloride with dithiothreitol, and alkylation with iodo-acetic acid, as described in Paper 9. Digestion with trypsin (TPCK, Worthington) was performed in 0.1 M NH₄HCO₃ (Paper 9). Cyanogen bromide degradation was done according to Steers et al (1965) and amino acid analyses according to Spackman et al (1958) and Moore (1963).

At fluorescence microscopy epi-illumination with narrow band excitation for the two wave-lengths (Hijmans et al 1971) was used.

CLINICAL MATERIAL

Nilsson et al (1961 and 1965) assessed the incidence of haemophilia and Silver (1973) the incidence of von Willebrand's disease in Sweden. In family studies in the present investigation the same family numbers and identification symbols (initials) of the patients were used as in previous publications. In addition, the pedigrees of some families with von Willebrand's disease not described previously are given in Appendix 2.

The rest of the clinical material consists of patients treated at different departments of Malmö general hospital.

I. CONGENITAL DISEASES WITH LOW FACTOR VIII ACTIVITY

The purpose of this part of the investigation, which was started in 1970, was to study a Swedish clinical material consisting of patients with low factor VIII activity with the aid of immunological techniques as well as new methods for assaying platelet adhesiveness, with proteinchemical methods and later also with Ristocetin-induced platelet aggregation. It was intended to include in the material cases of typical classical haemophilia A and cases diagnosed as von Willebrand's disease according to conventional criteria.

During the investigation several observations bearing on diseases with low factor VIII activity were made in other parts of the world. These observations will be commented upon with the results of the Swedish investigations in the Discussion of each section.

1. Haemophilia A

Background

The disorder in haemophilia A consists of an isolated reduction of factor VIII activity. Other components of the haemostatic system are normal. Factor VIII activity may be reduced to a varying extent with varying severity of the disease as a result. According to the level of the factor VIII activity, three forms of haemophilia A are recognised, viz. severe haemophilia A with an activity of less than 1 %, a moderate form with 1-4 %, and a mild form with 5-25 % of normal (Prinkhous and Graham 1954, Nilsson et al 1961). The mild form was first described by Graham et al (1953). The affected members of a haemophilic family have haemophilia of equal severity, and they have the same level of factor VIII activity (Quick and Hussey 1952, Biggs and McFarlane 1958, Pitney and Arnold 1959, Nilsson et al 1961 and 1965, Lewis et al 1963).

Statistical treatment of the data from the Swedish investigations (Nilsson et al 1961 and 1965) suggests that factor VIII activity in haemophiliacs is regulated by an abnormal X-chromosomal gene, which is specific for each particular haemophilia family (Roberts 1971).

The molecular defect underlying the coagulation disorder in haemophilia has long been discussed. After demonstration of the anti-haemophilic globulin (Patek and Taylor 1937) it seemed reasonable to assume that the defect consisted of deficiency or absence of this

globulin in haemophiliacs. Tocantins (1954), however, felt that it was due to an excess of a specific lipid inhibitor. A similar opinion was expressed later by Mammen (1963). Another feasible explanation is that a factor VIII molecule with a defective structure has little or no clot-promoting activity. In view of the specific mutation in every haemophilic family this theory appears attractive. Many examples of mutations causing changes in the primary structure of a protein, e.g. in the form of amino acid substitutions, are known, e.g. haemoglobino-pathies. It might be possible to demonstrate such a defective factor VIII molecule by immunological methods.

The early literature contains many conflicting observations regarding the presence of cross reacting factor VIII related antigenic material in haemophilia plasma. This is certainly because the heterologous antisera were far from specific owing to impure factor VIII preparations for immunisation. With the aid of different factor VIII neutralisation tests some authors (Shanberge and Gore 1957, Berglund 1962, Piper und Schreier 1964, Bennett and Huehns 1970), but not others (McLester and Wagner 1965, Uszyński 1966) made it probable that haemophilia plasma as well as serum (Piper und Schreier 1964) contains an antigenic form of factor VIII. But no investigation had succeeded in demonstrating a precipitation reaction between factor VIII and a heterologous antibody before Zimmerman et al (1971), possibly with the exception of Vainer et Caen (1964) and Uszyński (1967). Zimmerman et al, however, used a specific antiserum. It is obvious from their investigations that haemophiliacs have a factor VIII related material.

With the aid of human antibodies against factor VIII, however, such material could most often not be demonstrated in haemophilia plasma (Abildgaard et al 1967, Hoyer and Breckenridge 1968, Feinstein et al 1969, Denson et al 1969) but it could be in serum (Abildgaard et al 1967, Hoyer and Breckenridge 1968).

Results

The patients were divided into the three groups of severe, moderate and mild haemophilia, according to the criteria given above. The usual pattern was observed that the disease was of the same severity in affected members of the same family (Table I, Paper 1).

The specificity of the heterologous antibody is clear from Papers 1, 3 and 7. The strongest evidence that the antibody is directed against a protein related to factor VIII is that no precipitation

line is obtained on immunodiffusion with plasma from a patient with severe classical von Willebrand's disease. The antibody inhibits factor VIII activity in normal plasma, but not completely (Papers 1 and 3).

The heterologous antiserum detected an antigen both in normal and haemophilic plasma. Crossed immunoelectrophoresis of cryoprecipitate showed that the antigens in normal and haemophilic cryoprecipitate were identical and had the same electrophoretic mobility. Quantitative comparison of the amount of antigen in normal and haemophilia A plasma was therefore possible.

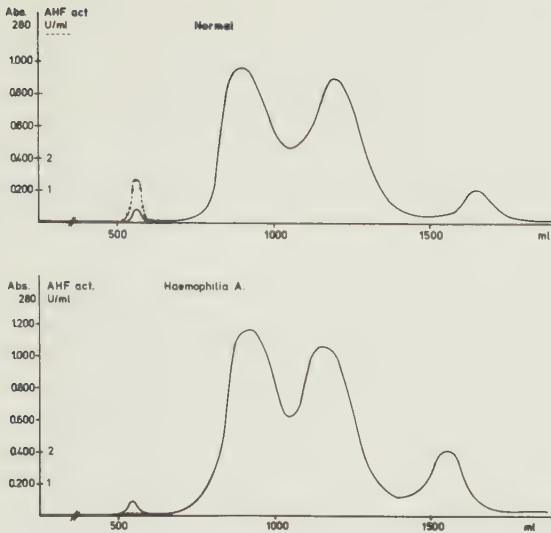
The examination revealed factor VIII related antigen in all the 44 patients. 27 had severe, 9 had moderate and 8 had mild haemophilia A. The mean value found for factor VIII antigen was higher in the haemophiliacs than in the controls ($p < 0.01$). There was no definitely significant difference between the patients with severe, moderate respectively mild haemophilia ($p > 0.01$).

Identity by crossed immunoelectrophoresis was also shown between the antigens in normal plasma and in plasma from patients with haemophilia A and a circulating anticoagulant. On quantitative examination we found values of the same order in haemophilia patients with anticoagulant as in those without (Table II, Paper 1).

In the inhibitor-neutralising test the inhibitor used was obtained from a patient with severe haemophilia A, who had developed an anticoagulant. Only four haemophiliacs showed any ability to neutralise the antibody. They belonged to the same family and had the mild form of haemophilia A.

In the course of this study the factor VIII related protein was isolated individually from RK and EK, fam 22, LT, fam 75, LS, fam 76, all with severe haemophilia A, and from LJ and BJ, fam 27, with moderate haemophilia A, as well as from two additional patients with severe haemophilia A and not included in Table I, Paper 1. In both severe and moderate haemophilia it had the same behaviour on gel chromatography according to van Mourik and Mochtar (1970) as normal factor VIII related protein, but it lacked factor VIII activity almost entirely (Fig. 1). The yield of the protein from haemophilic plasma was the same as from normal plasma and could be roughly predicted from the amount of antigen in the respective plasmas.

Fig. 1



Sepharose 6 B chromatography of cryoprecipitate from normal plasma (above) and haemophilic plasma (below).

Discussion

Investigation of a Swedish haemophilia material with heterologous antiserum clearly demonstrated that haemophilia A plasma contains a factor VIII related antigen. The results are essentially in agreement with those reported in the original publications by Zimmerman et al (1971) and Stites et al (1971) and later publications from various parts of the world (Meyer et al 1972, Hoyer 1972a, Bouma et al 1973). Our antiserum could not detect any difference between the antigen in normal plasma and in haemophilia plasma, as also reported by Zimmerman et al (1971), Bouma et al (1972), Meyer et al (1972) and Hoyer (1972a).

Zimmerman et al (1971) found no difference in the amount of antigen between normals and haemophiliacs, while the present study, like those reported by Hoyer (1972a) and Bouma et al (1973), showed significantly higher values in haemophiliacs than in normals. Factor VIII antigen increases in reactive processes (see section II), and the higher values in haemophiliacs can probably be explained by reactive processes being more common in these patients than in normals. However, there was no definitely significant difference between severe, moderate and mild haemophilia, respectively, which was probably because our groups of patients with moderate and mild haemophilia were

small. Other investigators have not systematically divided their material into these groups.

The reason why certain patients with haemophilia A are apt to develop anticoagulants after infusion of plasma or factor VIII concentrates might be that these patients, unlike other haemophilia patients, are deficient in factor VIII antigen in the plasma. But we found a normal antigen content in patients with anticoagulants like Zimmerman et al (1971), Stites et al (1971), Meyer et al (1972) and Denson (1973). Prentice and Forbes (1972) found even higher values in patients with anticoagulant.

Biochemical studies produced further evidence of a structurally defective factor VIII protein in haemophilia A. Hershgold et al (1967) were the first to present some evidence from gel filtration that an inactive protein might be present in haemophilic plasma. van Mourik and Mochtar (1970) and Bouma et al (1972) found identical OD patterns in chromatograms of cryoprecipitate from normal plasma and from plasma of three patients with severe haemophilia A. Using the method of van Mourik and Mochtar we isolated factor VIII protein individually from 8 patients with haemophilia A of varying severity. Taken together these studies thus showed that a biologically defective factor VIII protein or protein complex occurs in all the types of haemophilia A. An inactive factor VIII protein from haemophilic plasma has been isolated also by Shapiro et al (1973) and Bennett et al (1973). Biochemical comparisons of the normal and the defective proteins are further presented in section III.

In the present investigation only four patients with mild haemophilia from the same family could neutralise a human inhibitor to factor VIII. The other patients with mild haemophilia could not do it, however. Thus neutralising ability does not seem to be correlated with factor VIII activity. Others have reported that also some patients with severe or moderate haemophilia A can neutralise the inhibitor (Denson et al 1969). The four patients in the present investigation might have a specific mutation preserving some antibody neutralising structures in the molecule, as well as structures responsible for factor VIII activity. The results of antibody neutralising studies may, of course, depend also upon the specific human antibody used. Human antibodies from different sources may differ in specificity. It has been shown that human antibodies have a different specificity and bind in a different way to the factor VIII protein than rabbit antibodies (Hoyer 1972b).

2. von Willebrand's disease (Papers 2-5)

Background

Nilsson et al (1957a,b) established the two original criteria for von Willebrand's disease (vWd), viz. a prolonged bleeding time and a low factor VIII activity. Nilsson et al (1959) later showed that patients with vWd lack a plasma factor occurring not only in normal plasma, but also in haemophilia plasma. This so-called von Willebrand factor (vWf), is not present in pure fibrinogen fractions or in platelets. It not only corrects the prolonged bleeding time in patients with vWd, but also apparently stimulates the production of factor VIII activity. The plasma fraction I-0 (Blombäck and Blombäck 1956), prepared from haemophilia plasma and thus devoid of factor VIII activity increases the factor VIII activity when given to patients with vWd (Nilsson et al 1959). The vWf appears to be closely related to factor VIII and occurs in factor VIII rich plasma concentrates (Nilsson et al 1959, Bennett and Dormandy 1966).

It was formerly not known how a plasma factor could affect the primary haemostasis and shorten the bleeding time. The basis of primary haemostasis is the formation of a platelet plug. Borchgrevink (1961) found decreased platelet adhesiveness in vivo in patients with vWd by comparing the platelet count in venous blood and in blood from a capillary lesion. A reliable in vitro test for platelet adhesiveness was devised by Hellem (1960).

Hellem's original method, which used citrated blood, did not distinguish normal persons from patients with vWd (Hellem 1960, Cronberg et al 1966). Salzman (1963) used native blood and a quicker flow and could then demonstrate decreased platelet adhesiveness to glass in vWd. His results have been confirmed by many groups. Modifications of Salzman's method, all with a rapid blood flow, have also proved diagnostically useful (O'Brien and Heywood 1967, Bowie et al 1969, Hellem 1970), though they are technically difficult and not free from methodological errors (Bowie and Owen 1971, Collier and Zucker 1971). Common to all methods for measuring platelet retention in a glass bead column (platelet adhesiveness) and capable of distinguishing normal persons from patients with vWd is that they use a rapid flow of blood (Cronberg and Holmberg 1973).

The reduced platelet adhesiveness in vWd, like the bleeding time, can be normalised by infusion of fresh platelet-poor plasma (Salzman 1963). Such normalisation can be demonstrated in vitro by mixing the

patient's blood with normal plasma, haemophilia A plasma or cryoprecipitate (Salzman and Britten 1964, Meyer et Larrieu 1970, Weiss and Rogers 1972, Bouma et al 1973). The factor which causes the normalisation is missing in vWd plasma (Meyer et Larrieu 1970, Bouma et al 1973). Furthermore, Bouma et al (1972) found that a purified factor VIII protein fraction from both normal and haemophilic plasma normalised the decreased platelet adhesiveness in vWd.

Further knowledge about vWd was obtained after monospecific antisera against factor VIII had become available (see preceding section). Haemophilia A could then be differentiated immunologically from vWd (Zimmerman et al 1971, Stites et al 1971). Howard and Firkin in 1971 made another important discovery, when they studied the mechanism of platelet aggregation with the antibiotic Ristocetin. Their studies suggested that Ristocetin had its effect on aggregation by specifically interacting with a plasma factor missing in patients with vWd. Meyer et al (1973) and Weiss et al (1973b) showed that this plasma factor was identical with factor VIII.

The manifestations of bleeding, like the laboratory findings in vWd can vary widely (v.Willebrand 1926, Abildgaard et al 1968). The diagnosis of vWd must therefore be made partly on the basis of the patient's history, partly on several laboratory examinations. The criteria for the diagnosis vWd were the same in this study as in Silver's (1973) work:

In probands:

1) History of bleeding tendency with severe or frequently recurring abnormal bleedings that cannot be explained by any other disease.

2) Reduced factor VIII activity (<60 %).

3) Bleeding time according to Duke more than 5 min or according to Ivy more than 12 min.

In most cases diagnosis was further confirmed by:

4) Occurrence of similar bleeding disease in the patient's family.

5) Decreased platelet adhesiveness according to Salzman.

6) Retarded increase in factor VIII activity and shortening of the bleeding time after infusion of fraction I-0.

In relatives:

In relatives of the probands the diagnosis of vWd was made if

they met two of the three first mentioned criteria (1-3).

A large portion of the recorded Swedish families with vWd, diagnosed according to these criteria, was examined for factor VIII antigen in plasma. The results are given partly in Papers 2 and 3.

Results

In most of the families the affected members had a decreased amount of factor VIII antigen, determined with a monospecific rabbit anti-serum. In some families, however, the affected members had a normal antigen content. The first type was called vWd, type I; the second vWd, type II.

vWd, type I, is described in section A and a variant of vWd, type I, in section B. Some of the families with vWd, type I, are given in Table I, Paper 2. Further families have been examined since the publication of this work. 39 families with vWd, type I, are shown in appendix 1a. The family numbers are those used by Silwer (1973) up to and including family 105. The pedigrees have also been given by Silwer but some have been supplemented and new ones have been included (appendix 2a).

vWd, type II, is described in sections C and D and in Papers 2 and 3. The data available in August 1974 are summarised in appendix 1b. Also there the family numbers are those used by Silwer, but some families have been supplemented (appendix 2b). 33 patients belonging to 12 families had a vWd-like disease but with a normal amount of antigen. In nine of the families the factor VIII activity was low. Under C 1 seven such families are described, in which the inheritance seems to be sex-linked. Section C 2 (Paper 5) deals with a case of vWd, type II, from a family, which also includes cases of typical haemophilia A. Section C 3 describes a family, in which the sick members have low factor VIII activity and normal amount of factor VIII antigen, but the discrepancy between activity and antigen was undoubtedly less than in group C 1. Besides, the inheritance is obviously autosomal. This family is therefore treated separately. In three families the amount of antigen in the affected members was normal, but, in addition, also factor VIII activity was normal. The patients in these families were nevertheless regarded as having vWd, as the other criteria of vWd were present (see below). This variant is dealt with in section D.

A. von Willebrand's disease, type I (Holmberg and Nilsson 1972)

Low factor VIII antigen, low factor VIII activity
(appendix 1a and 2a)

39 families with all together 100 affected members examined were assigned to type I. Five of the 100 had not reduced content of factor VIII antigen at the time of the examination, namely III:42, fam. 42, II:2, fam. 89, II:2, fam. 92, I:1, fam. 103 and III:1, fam. 105. A further three had borderline values (III:40, fam. 42, P.K., fam. 139 and B.D., fam. 141). Of these eight, two were pregnant (III:42, fam. 42 and P.K., fam. 139). In III:42, fam. 42 the value later rose to very high levels when she developed toxæmia of pregnancy. II:2, fam. 92 and III:1, fam. 105 used oral contraceptives. One patient, II:2, fam. 89 was of advanced age (85 years). I:1, fam. 103, had gastric cancer with metastases at the time of the examination.

The correlation between factor VIII antigen and the bleeding time in vWd, type I, is shown in Table I. The correlation between a low antigen content and a long bleeding time is obvious. Judging from appendix 1a, on the other hand, there is no clear correlation between factor VIII activity and factor VIII antigen in the group. The patients with the lowest values for antigen, however, also had the lowest values for activity.

49 of 58 patients examined had a low platelet adhesiveness with Salzman's method. 4 cases with severe vWd, type I (III:1, fam. 10, II:1, fam. 11, IV:2, fam. 12 and III:2, fam. 135) had very low platelet adhesiveness with Bowie's system.

Table I

<u>Bleeding time, min.</u>	Factor VIII antigen %			
	<u>0-7</u>	<u>8-20</u>	<u>21-50</u>	<u>>50</u>
Duke >30	16	4	0	0
Duke <30	1	13	16	1
Ivy >30				
Ivy <30	1	8	25	3
Number of patients				

The correlation between bleeding time and factor VIII antigen
in vWd, type I

Ristocetin-induced platelet aggregation was investigated in 4 patients. In patients III:1, fam. 10 and II:1, fam. 11, Ristocetin was added to the platelet-rich plasma of respective patient to varying final concentrations of Ristocetin. Addition of Ristocetin to a concentration of 1.2 mg/ml, which aggregated the platelets in normal PRP, failed to produce aggregation in these two patients. In patient II:1, fam. 11, it occurred, however, at a concentration of 1.5 mg/ml.

In patients IV:1, fam. 58 and III:2, fam. 135, Ristocetin aggregation was tested quantitatively according to Weiss et al. It was markedly reduced in both of them (7 respectively <5 %).

Sephacrose 6 B chromatography of cryoprecipitate was performed on two patients, namely II:1, fam. 11 and III:4, fam. 14. The results are given in Fig. 2. In II:1, fam. 11 no protein and no factor VIII activity could be demonstrated in the void volume and in III:4, fam. 14 the material in the void volume had very low absorbance at 280 nm (0.002) and a low factor VIII activity (14 %).

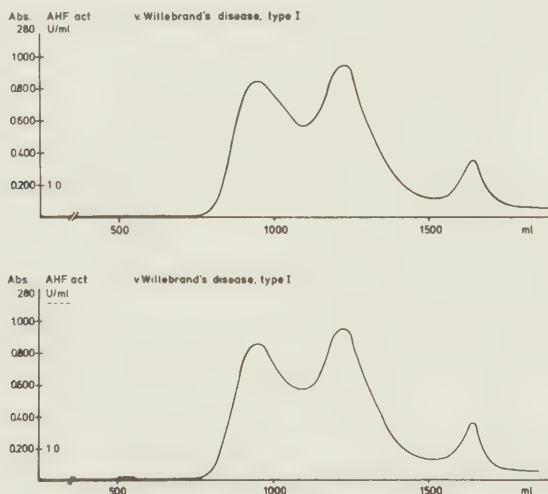


Fig. 2 Sepharose 6 B chromatography of cryoprecipitate from plasma of patients with vWd, type I. II:1, fam 11 above, III:4, fam 14 below.

Infusions. Factor VIII concentrate (human fraction I-0, "AHF KABI") was in this study infused in six patients with vWd, type I (III:1, fam 10, II:1, fam 11, IV:2, fam 12, II:3, fam 14, IV:5, fam 17 and II:1, fam 130) (see Paper 3). After infusion of fraction I-0 five of the six patients showed a progressive increase of factor VIII activity. In the sixth (II:3, fam 14) it did not increase, but persisted at a high steady level for more than 24 hours. The bleeding time according to Duke returned to normal. The factor VIII related antigen never increased to a level higher than the immediate posttransfusional value but fell progressively. At a certain time after the infusion there was therefore a dissociation between factor VIII activity, which was high, and the factor VIII antigen content, which was low. This dissociation appeared clearest in the severest cases where the content of antigen before the infusion was very small. In the milder cases with a certain initial concentration of factor VIII antigen, the difference between the curves for activity and antigen was not so striking.

Inheritance. In ten families (6, 14, 17, 33, 42, 58, 122, 131, 135, 143) the disease was transmitted from father to son. In families 12 and 17 two patients had consanguineous parents. Table II gives the offspring of affected men and of affected women.

Table II

Offspring of affected men				Offspring of affected women			
Sons		Daughters		Sons		Daughters	
Healthy	Affected	Healthy	Affected	Healthy	Affected	Healthy	Affected
23	30	17	29	38	34	26	39

Offspring of affected men and women in vWd, type I

In nine families (6, 14, 23, 42, 58, 60, 131, 135, 142) both parents of the proband were available for determination of factor VIII antigen. In seven of them the result was clear: one parent had an abnormal level of factor VIII antigen (11-36 %), and the other parent had a normal level (80-105 %). In family 23 the father of the proband (II:5), who had been regarded by Silwer as a carrier of the disease, had a normal antigen value, though in the lower part of the normal range, while the mother (II:15) had a slightly subnormal value (47 %). II:5 had factor VIII activity 22 % when tested in 1959 but

135 % when tested in 1972! The corresponding factor VIII activity in II:15 was 100 respectively 70 %. In family 135 the asymptomatic father (II:1) had a low antigen value (37 %), while the mother (II:2) was normal at most examinations (49, 66, 74 % respectively). Factor VIII activity was normal in both (94 respectively 95 %).

B. Variant of von Willebrand's disease, type I

Low factor VIII antigen, normal factor VIII activity

Paper 4 describes a girl with a severe bleeding disease in the form of life-threatening menorrhagia. She filled the criteria for vWd with the exception that she had normal factor VIII activity on repeated occasions. This finding contrasted with her very low content of antigen and long bleeding time. Her platelet adhesiveness according to Salzman was low, and she showed a progressive rise in factor VIII activity after infusion of factor VIII concentrate. Ristocetin-induced platelet aggregation was only slightly decreased. Her platelets were normal. The investigation of the molecular defect in this patient is described in Paper 4. The factor VIII activity of the patient's plasma as well as of normal plasma was eluted at the void volume of a column with Sepharose 6 B. The factor VIII activity of normal plasma was eluted at the void volume also of a column with Sepharose 2 B, but the factor VIII activity of the patient's plasma was retarded in such a column.

Both parents of the patient had slightly subnormal values for factor VIII antigen and reduced platelet adhesiveness according to Salzman. They were first cousins.

C. von Willebrand's disease, type II (Holmberg and Nilsson 1972 and 1973)

Normal factor VIII antigen, low factor VIII activity
(appendix 1b and 2b)

1. Probably X-chromosomal inheritance

This type of the disease was reported in Papers 2 and 3. In families 28, 55, 67, 88, 100, 107 and 137 factor VIII activity was low in the affected members and much lower than the content of factor VIII anti-

gen (borderline in III:6, fam. 28 and II:9, fam. 100; also these, however, had a much higher content of antigen than activity).

The lowest factor VIII activity in group C 1 was 10 %, but also values up to the lower limit of a normal range were seen. The mean value found for factor VIII activity in the affected male members of these families was 33 %; in the females, 46 % ($0.01 < p < 0.05$).

Bleeding time according to Ivy's method was determined repeatedly (3 times or more). In 20 patients the bleeding time was prolonged, but was normal in 6. The bleeding time according to Duke's method, on the other hand, was only occasionally prolonged.

Platelet adhesiveness with Salzman's method was decreased in 9 of 17 cases. Three of the 8 patients in whom Salzman's test was normal had a decreased adhesiveness according to Bowie's method (see appendix 1b).

In II:4, fam. 28, III:10, fam. 55 and III:7, fam. 100, the platelets were studied further. All had a normal platelet aggregation with ADP, adrenaline and collagen.

Ristocetin-induced platelet aggregation was examined quantitatively in III:7, fam. 100, and was normal (130 %). II:4, fam. 28, had a normal aggregation in PRP at a final Ristocetin concentration of 1.2 and 1.5 mg/ml.

Sephacrose 6 B chromatography of cryoprecipitate. Cryoprecipitate from 400 ml plasma from III:3, fam. 28, was chromatographed on dextran containing Sepharose 6 B. A typical protein peak was obtained at the void volume (abs 280 nm in the best fraction was 0.029). The factor VIII activity in the void volume material was lower (27 %) than when cryoprecipitate from normal plasma was used as a starting material.

Infusions. Factor VIII concentrate (human fraction I-0, "AHF KABI") was in this study infused to two patients (III:3, fam. 28 and III:7, fam. 100). No retarded increase of factor VIII activity was recorded, but the bleeding time was shortened. Factor VIII antigen gradually returned to the preinfusion level (Paper 3).

Inheritance. There was no instance of transmission of the disease from father to son in families 28, 55, 67, 88, 100, 107 or 137. The sex proportions among the 26 affected members were 10 men and 16 women. Table III gives the offspring of affected men and of affected women.

Table III

Offspring of affected men				Offspring of affected women			
Sons		Daughters		Sons		Daughters	
Healthy	Affected	Healthy	Affected	Healthy	Affected	Healthy	Affected
11	0	2? ^x	9	13	10	11	5

Offspring of affected men and women in vWd, type II (C 1)

x) IV:5 and IV:6, fam. 28, see appendix 1b. Age at examination 6 and 2 years respectively. The large discrepancy between activity and antigen in these two girls suggests that they are affected.

2. Co-existent with typical haemophilia A in the same family

Paper 5 describes a girl with a severe bleeding disorder. Her disease was characterised by bleeding from the mucous membranes and the skin as well as by joint bleedings. Her karyotype was XX. She had laboratory values typical of severe vWd: factor VIII activity 5 %, bleeding time according to Duke 30 min, platelet adhesiveness according to Salzman 7-18 %. Her bleeding time was normalised by infusion of factor VIII concentrate (AHF KABI), but no retarded increase in factor VIII activity was ever recorded. She had a normal amount of factor VIII antigen and a normal Ristocetin-induced aggregation in PRP (at final concentrations of Ristocetin of 1.2 and 1.5 mg/ml). The platelet response to ADP, adrenaline and collagen was normal.

Her mother had a somewhat low factor VIII activity and a slightly prolonged bleeding time. Her father was normal. A male cousin had typical haemophilia A with 2 % factor VIII activity and the other tests normal. There was also another case of haemophilia among the male members of the family. Four females had low factor VIII activity, but otherwise no signs of vWd, for which reason they were identified as carriers of haemophilia A.

3. Autosomal dominant inheritance

Family 5 was first classified as vWd, type I, as low values for antigen were obtained, when the patients' cryoprecipitates were examined. When antigen was retested directly on plasma, the patients were found to have normal values. III:5 is the father of IV:1, and both

have had rather severe bleeding symptoms. III:17, the mother of IV:1, is healthy. Platelet adhesiveness according to Salzman and Ristocetin-induced aggregation were subnormal in the patients. Infusion of factor VIII concentrate normalised the bleeding time. In III:5 a slight progressive increase in factor VIII activity was noted, and in IV:1 factor VIII activity persisted at a high level 24 hours after the infusion. The antigen decreased somewhat faster than the activity. Sepharose 6 B chromatography of cryoprecipitate from III:5 gave a void volume peak of very low absorbance at 280 nm (0.004) and with low factor VIII activity (20 %) (compare vWd, type I).

D. von Willebrand's disease, type II, with normal factor VIII activity

Normal factor VIII antigen, normal factor VIII activity

Three families (75, 138, 146) were assigned to the group. Patient II:1, fam. 75, had low platelet adhesiveness according to Salzman, somewhat low Ristocetin-induced aggregation and prolonged bleeding time, despite normal factor VIII activity and antigen. Patients I:2 and II:2, fam. 138 are mother and son, both with mild bleeding symptoms, reduced platelet adhesiveness and without signs of any other bleeding disorder. Patient Y.A., fam. 146, had conspicuous bleeding symptoms. Factor VIII antigen and activity were normal (although in the lower range), the bleeding time was prolonged and the platelet adhesiveness according to Salzman was reduced. Infusion of factor VIII concentrate was followed by a shortening of the bleeding time and a retarded increase in factor VIII activity.

Discussion

Several workers have felt that the concept of vWd in fact comprises a group of related disorders (Bowie et al 1967, Weiss 1968, Edson 1970). Zimmerman et al (1971) found that the disease was uniform in so far as the factor VIII antigen content in all their patients was low. Holmberg and Nilsson (present work, Paper 2), however, demonstrated that a group of patients with the diagnosis of vWd was heterogeneous, since a few of them had a normal level of factor VIII antigen. The existence of genetic variants of vWd has then been recognised by several authors (see Table IV).

In my experience the special type of vWd is easily recognised in every separate family. If one sick patient in the family has a low level of factor VIII antigen, then other sick members also have a low level. It is essential, however, to examine the patients under basal conditions, because reactive processes raise the factor VIII content in healthy persons as well as in patients with vWd (Paper 7, present work, Silwer 1973). Factor VIII may also rise in advanced age. Bearing these observations in mind no families were seen in which some of the affected members had a low antigen content, while other affected members had a normal content. Such families have since been reported by Meyer et al (1974) and Thomson et al (1974). The amount of antigen admittedly could vary markedly between affected members of the same family, but always within the subnormal range. Judging from appendix 1a, the content of antigen in vWd, type I, varies largely with the severity of the disease. All patients with the severe form, except III:9, fam. 58, had very low values. It is well known that the expressivity of vWd can vary within one and the same family (Silwer 1973).

Factor VIII activity in plasma is normally linked to a large macromolecule (Ratnoff et al 1969). This macromolecule has several well-defined properties: 1) it acts in intrinsic plasma coagulation = factor VIII activity; 2) it precipitates with a specific rabbit anti-serum (Paper 1) = factor VIII antigen; 3) it probably interacts with platelets in primary haemostasis and is necessary to keep bleeding time and in vitro platelet adhesiveness normal (Bouma et al 1972) = von Willebrand factor (vWf) activity; 4) it aggregates normal and vW-platelets in the presence of Ristocetin (Howard and Firkin 1971, Weiss et al 1973b) = Ristocetin cofactor (Rcof) activity.

In the patients with vWd, type I, the content of factor VIII protein was most probably really reduced. On gel filtration according to van Mourik and Mochtar (1970) little or no factor VIII protein was obtained when cryoprecipitate from type-I-vWd-patients was used as a starting material. It is less likely that the patients should have an abnormal, poorly cross-reacting protein with a reduced tendency to be cryoprecipitated. The ratio between the amount of antigen in the plasma and antigen in the cryoprecipitate in these patients was, as a rule, the same as in normal persons (Paper 2) (but compare Kernoff et al 1974 and the patients of section C 3, who seem to have a protein with less tendency to cryoprecipitate).

A reduction of the content of factor VIII protein in plasma

causes a reduction of all the functions of the substance: factor VIII activity, vWf activity and Rcof activity. This was recorded also in patients with vWd, type I: low factor VIII activity, as a rule very long bleeding times, low platelet adhesiveness and reduced or absent aggregation with Ristocetin. The striking correlation between factor VIII antigen and the bleeding time suggests that factor VIII protein has a direct effect on the primary haemostasis. A certain minimum amount of the protein is necessary for the formation of a platelet plug in normal time. If the amount of the protein present is only small, the plug will take a longer time to form.

The ratio of affected respectively healthy sons and daughters to affected men and women with vWd, type I, is such that an autosomal and dominant mode of inheritance is unequivocal. There is no convincing evidence that any of the patients is homozygous for an abnormal gene. The observed frequency of consanguineous marriages in two of the families was perhaps a little higher than expected. But none of the parents in those two families were available for examination for factor VIII antigen. The results in nine families, in which both parents of the proband were examined for factor VIII antigen, are compatible with dominant inheritance in all instances, but with a varying expressivity. It cannot be excluded with certainty, however, that III:12, fam 23 and III:2, fam 135 were homozygous. The patient described in Paper 4 was most probably homozygous for an abnormal autosomal gene. The mutation that occurred in her family differed clearly from those in the other families with vWd, type I.

The retarded increase in factor VIII activity after infusion of factor VIII concentrate has been regarded as a diagnostic sign of vWd (Nilsson et al 1959, Cornu et al 1960), but it is sometimes difficult to demonstrate in patients with mild vWd (Strauss and Bloom 1965, Ingram et al 1971, Veltkamp and Tilburg 1974). In the patients with type I this response was generally recorded.

The dissociation of activity and antigen in the transfused vWd-patient, type I, was described by Bennett et al (1972) and independently by Holmberg and Nilsson (Paper 3). Bloom et al (1973a) stated that the newly synthesised factor VIII activity lacking antigen could be recovered in low molecular weight fractions, but this was not verified by Weiss and Sussman (1974) and Ekert et al (1974). The patient described in Paper 4 had a coagulation status, which closely resembled that in an ordinary vWd patient 24-48 hours after an infusion. From the gel chromatography on Sepharose 2 B and 6 B in that

patient it can be concluded that a high molecular weight factor VIII can be formed despite abnormal autosomal genes and in our patient even without the stimulation by an infusion. The patient could not, however, make such a very high molecular weight factor VIII as is present in normal plasma. Thus the gene product(s) of normal autosomal genes seem to be required for the proper formation of the very large molecule with the normal antigenic determinants. This very large molecule is probably necessary for von Willebrand factor activity.

Theoretically, one might imagine other explanations of vWd than a decreased content of the very large factor VIII molecule in the plasma. Qualitative defects, which selectively affect one or more of the functions of the molecule might produce diseases, closely resembling classical vWd (=type I). The variants so far described are illustrated in Table IV.

Affected members of the families assigned to group C 1 had a normal antigen content, but low activity and prolonged bleeding time according to Ivy. On the other hand, as a rule, the bleeding time according to Duke was not prolonged and the platelet adhesiveness was not so regularly reduced as in vWd, type I. The vWf activity was thus not so low as in type I. There was no example of male to male transmission (Table III). Pedigrees from families 28, 55 and 100 suggested X-chromosomal heredity (Paper 2). In the other families of group C 1 the mode of inheritance could not be determined. The ratio of affected respectively healthy sons and daughters to affected men and women does not speak against X-chromosomal inheritance.

The disease in families 28, 55 and 100 might thus equally well be regarded as a variant of mild haemophilia. At least in family 100 the factor VIII activity was clearly lower in the males than in the females, and the bleeding time in the females was often normal.

Certain features differentiate it, however, from haemophilia: 1) a large proportion of symptomatic females suggests that the disease is often manifest even in heterozygotes (dominant); this is unusual in haemophilia, even if carriers of mild haemophilia may have values for factor VIII activity just as low as carriers of severe haemophilia (Graham et al 1953); 2) a disorder of the primary haemostasis with prolonged bleeding time and reduced platelet adhesiveness. The bleeding time and platelet adhesiveness are generally regarded as normal in haemophilia. Ikkala (1960) found a transient prolongation of the bleeding time (Duke) in the bleeding phase in only two haemo-

Table IV

Act +	Act -
Ant +	Ant +
vWf +	vWf +
Rcof +	Rcof +
<u>Normal</u>	<u>Classical haemophilia A</u>

von Willebrand's disease

A. Act - Ant - vWf - Rcof -	B. Act + Ant - vWf - Rcof (+)	C 1. Act - 2. Ant + vWf - Rcof +
<u>Classical vWd</u> (autosomal dominant), type I	Autosomal recessive Holmberg et al 1974	<u>vWd, type II</u> X-chromosomal Holmberg & Nilsson 1972, Bouma et al 1973? Autosomal dominant Ratnoff & Saito 1974 Firkin & Koutts 1974
C 3. Act - Ant + vWf - Rcof -	D. Act + Ant + vWf - Rcof -	
<u>vWd, type II</u> Autosomal dominant Holmberg 1974 Stableforth et al 1974 (her?) Peake et al 1974 (her?) Kernoff et al 1974 (Rcof?)	<u>vWd, type II</u> Autosomal dominant Thomson et al 1974 Peake et al 1974 Firkin et al 1973 (her?) Holmberg 1974 (her?)	
Types described by other workers:		
E. Act - Ant - vWf + Rcof (+)	F. Act - Ant - vWf + Rcof -	G. Act + Ant - vWf + Rcof ?
Autosomal Bloom et al 1974 Veltkamp & Tilburg 1974 (Rcof?)	Autosomal dominant Thomson et al 1974	Autosomal Veltkamp & Tilburg 1973 (Clinically healthy heterozygotes)

Variants of vWd hitherto described (Sept 1974)

Act = F VIII activity
Ant = F VIII antigen
vWf = von Willebrand factor activity
Rcof = Ristocetin cofactor activity

philiacs and in two patients over 45. Nilsson et al (1961) found no patients with mild haemophilia A with a prolonged bleeding time. Salzman (1963) reported platelet adhesiveness to be normal in haemophilia, while Bouma et al (1973) found it to be somewhat reduced in some haemophiliacs (Bowie's method). In our own experience 4 haemophiliacs out of 29 had a slightly prolonged Ivy bleeding time (modification Borchgrevink and Waaler 1958) (13-19 min), while it was normal in the rest. Values above the upper limit of the normal range (12 min) are occasionally seen also in the normal population (Cronberg 1968). Aspirin was excluded as a cause of the prolonged bleeding time. Haemophilia A co-existent with a platelet disorder (Chesney et al 1974) could also be ruled out.

Of special interest is the family described in Paper 5 (C 2). The co-existence of haemophilia A and von Willebrand's disease has been recognised earlier (Quick and Adlam 1963, Geiger and Rath 1963). Determination of factor VIII antigen was, however, not possible at that time, for which reason it is difficult to compare the present family with those of the other authors. Our patient, who has a normal amount of antigen, may be a double heterozygote for two different X-chromosomal mutations, affecting factor VIII. One of these is the same as that giving rise to moderate haemophilia A in her hemizygous cousin, the other one may be a new one of the same type as that giving rise to the vWd-like picture in the families in section C 1. As a matter of fact, the girl, who has 5 % activity, has more frequent and severe bleedings than her male cousin, who has 2 %.

Infusion of factor VIII concentrate in two patients in group C 1 (from fam 28 and 100) and in the patient described under C 2 shortened the bleeding time. No retarded increase in factor VIII activity could be demonstrated, which argues for the haemophilioid nature of the disease in these families.

A disease resembling that described in section C 1 was recognised by Egeberg (1965), but available data on that family are not complete. vWd with low factor VIII activity, normal factor VIII antigen, prolonged bleeding time and normal Ristocetin-induced aggregation was reported in 1974 by others (Firkin and Koutts 1974, Ratnoff and Saito 1974) and vWd with low factor VIII activity, normal factor VIII antigen, reduced platelet adhesiveness and prolonged - normal bleeding time also by Bouma et al (1973) (Ristocetin-induced aggregation not tested). The patients of Bouma et al may have been of the same type as ours. The patients of Firkin and Koutts and of Ratnoff and Saito

were claimed to have an autosomally inherited disease, but no details were given.

The family described in section C 3 resembled the families of section C 1 in some of the laboratory tests (low factor VIII activity and normal factor VIII antigen), but Ristocetin cofactor activity was decreased, and a retarded increase in factor VIII activity after infusion of factor VIII concentrate was recorded. Furthermore, the inheritance is no doubt autosomal dominant. The variant seems to be the same as that in some of the patients described by Kernoff et al (1974), Stableforth et al (1974) and Peake et al (1974), although these works do not give detailed information on heredity.

Family 5, group C 3 was more similar to the patients of group D than those of group C 1. However, the patients in group D had both normal factor VIII antigen and factor VIII activity, but they should nevertheless be regarded as having vWd because of their prolonged bleeding time, low platelet adhesiveness according to Salzman and retarded increase of factor VIII activity after infusion of factor VIII concentrate. No conclusion about the mode of inheritance could be made from the pedigrees of group D. Similar families have, however, been recognised by other authors (Firkin et al 1973, Thomson et al 1974, Peake et al 1974), who claimed that the inheritance was autosomal and dominant.

Besides the variants of vWd described in the present work another type has been reported by other authors. Bloom et al (1974), Veltkamp and Tilburg (1974) and Thomson et al (1974) have described a variant, in which the factor VIII molecule appears to be lacking in both factor VIII activity and normal antigenic determinants, but possesses vWf activity (Table IV). Ristocetin-induced aggregation was only moderately reduced in some such cases (Bloom et al 1974).

The four properties of the factor VIII molecule thus seem to be able to vary more or less independently of each other. The theoretical background to the variations of the function of the factor VIII molecule in relation to its structure is discussed further below (section III).

3. Vascular factor VIII in von Willebrand's disease and haemophilia A (Paper 6)

Background

von Willebrand's disease was once called vascular haemophilia (Schul-

man et al 1955) because it was associated with a decreased antihæmophilic factor (factor VIII) activity and a prolonged bleeding time. The latter was thought to be due to a defective vascular function. Later the deficiency in plasma of a bleeding factor (Nilsson et al 1957a) and abnormal platelet adhesiveness (Salzman 1963) received most attention. Both these findings have now been ascribed to a reduced plasma content of, or to a defect in, the factor VIII molecule (see preceding section).

In 1973(b) Bloom et al and Hoyer et al demonstrated the presence of factor VIII antigen on the vascular intima. Synthesis of factor VIII protein by fetal endothelial cells was reported by Jaffe et al (1973).

The aim of the studies in this section was to elucidate the role of vascular factor VIII in the diseases with low factor VIII activity. In contrast with other investigators, we used a direct immunofluorescent technique, which facilitated the tests for specificity.

Results

Biopsy specimens of superficial veins were obtained from 5 patients with vWd and 3 with hæmophilia A and studied for factor VIII antigen. 15 healthy men served as controls. In all the controls a bright specific fluorescence was observed in the intimal layer of the vein. Three patients with severe vWd, type I lacked the antigen in the intima. The fluorescence was, however, present in one patient with moderately severe vWd, type I, and in one patient with vWd, type II (the same patient as is described in Paper 5), as well as in 3 with classical hæmophilia A. In addition to the studies described in Paper 6, factor VIII concentrate was given to three patients with severe vWd, type I, without fluorescence in the vessel wall before the infusion (Holmberg, Mannucci, Nilsson, unpublished). One of these patients was M.A. (Paper 6), that is III:1, fam 10. She was subjected to gastric resection because of severe gastric hæmorrhage. She was given large doses of factor VIII concentrate before the operation, and the factor VIII activity rose to 250 % and the bleeding time became normal. The loss of blood at operation was not abnormally large. A venous biopsy from the region of the operation showed no specific fluorescence in the intima. Similar results were obtained in the other two patients.

Discussion

Judging from the studies in Paper 6, when factor VIII antigen is missing in plasma it is also missing in the vessel walls. If it can be detected in plasma, it can also be demonstrated in the vascular intima. Whether it is really produced by endothelial cells cannot be decided with the immunofluorescent approach. The infusion studies suggest that the presence of the antigenic protein in the intima is not due simply to adhesion to the endothelial surface.

It is difficult to assess the physiological significance of the antigen in the vessel compared with that in the plasma, because the defect in primary haemostasis can be promptly controlled by raising the plasma level of factor VIII. Howard et al (1974) found factor VIII antigen also in washed normal platelets, but not in platelets in von Willebrand's disease or thrombasthenia. In personal tests with the immunofluorescence technique (Holmberg, unpublished) the amount of factor VIII bound firmly to the platelet membrane of normal platelets was very small, which does not, however, exclude existence of the antigen in the interior of the platelet.

If our knowledge of the significance of the various sites of factor VIII protein is at present meagre, this is also true of our knowledge of the molecular mechanism underlying a supposed interaction between the protein and platelets. Purified bovine and porcine high molecular weight factor VIII can spontaneously aggregate human platelets in vitro (Forbes and Prentice 1973, Vermynen et al 1973, Griggs et al 1973). Human factor VIII can be modified in such a way with Ristocetin (see section I 2), and possibly also with enzyme neuraminidase, as to be capable of aggregating human platelets. It is known that the platelet membrane contains sialic acid residues (Maddoff et al 1964) as well as neuraminidase and sialyl transferase (Bosmann 1972). It has therefore been suggested that sialyl transferase in the platelet membrane forms an enzyme-substrate complex with exposed galactose residues either in factor VIII protein (Vermynen et al 1973) or in glycoprotein in the membrane of other platelets (Bosmann 1972), and thereby contributes to the adhesion-aggregation reaction of the platelets. If the factor VIII protein is missing or is defective, as it is in vWd, adhesion will also be defective. The mechanism would be analogous to the reaction postulated for adhesion of platelets to collagen (Jamieson et al 1971), but is still only hypothetical. Another possible mechanism of the interaction between factor VIII protein and platelets in vivo might be that under

certain circumstances the protein is adsorbed to the platelets, which would result in reduction of the surface charge of the platelets and by that decrease their mutual electrostatic repulsion (Seaman and Vassar 1966) and their repulsion from the endothelial surface.

II. FACTOR VIII IN DISEASE, IN PREGNANCY AND IN THE HUMAN FETUS

(Papers 7 and 8)

Background

Factor VIII activity may be low not only in congenital diseases, but also in some acquired disorders (Nilēhn 1962). Fibrinolytic states and intravascular coagulation may be accompanied by low factor VIII activity (Paper 7). More common, however, is the occurrence of high values of factor VIII activity in various diseases (Zetterqvist and v.Francken 1963, Larsson et al 1971) and in stress situations (Rizza 1961, Ikkala et al 1963).

Pregnancy is known to be accompanied by a rise in factor VIII activity, apparent from the second trimester (Strauss and Diamond 1963, Kasper et al 1964). Factor VIII activity in the newborn infant seems not to be correlated with that in the mother (Strauss and Diamond 1963). Data on factor VIII in the human fetus have long been lacking.

The aim of this part of the investigation was 1) to elucidate the relationship between factor VIII activity and factor VIII antigen in various diseases, which might be of value in the diagnosis or the understanding of factor VIII production and structure; 2) to examine factor VIII activity and factor VIII antigen in pregnant women and in newborn infants and fetuses. The data on factor VIII in the fetus might be useful for predicting a congenital disease.

Results

243 patients with cancer, liver disease, renal disease, myocardial infarction, burns, venous thrombosis and various haematologic disorders are reported in Paper 7. In all the disorders factor VIII antigen and activity were increased. Very large amounts of antigen were recorded especially in wide-spread cancer, liver disease, renal disease with uraemia, nephrotic syndrome, severe burns and in some cases of leukaemia, myelomatosis, macroglobulinaemia (Waldenström), haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. A less striking increase occurred in myocardial infarction. Patients with idiopathic venous thrombosis had somewhat elevated levels during remissions. Factor VIII antigen was often increased more than the activity. In some cases the level of antigen was more than twice or even three times the level of activity. In one patient with liver

necrosis the factor VIII protein could be isolated and its subunit structure determined (Paper 7).

Determinations of factor VIII in women during the three trimesters of pregnancy, during delivery and in the puerperium are shown in Table V. Five cases of toxæmia of pregnancy are also included. The correlation between activity and antigen is good in the first two trimesters ($r = 0.70$ respectively 0.69), but poor in the third ($r = 0.17$). For comparison 20 women using oral contraceptives were also examined (Table VI).

Table V

	<u>Patients</u>	<u>Factor VIII</u>	
		<u>Activity</u>	<u>Antigen</u>
1 st trimester	20	132.4±9.4	134.7±11.4
2nd trimester	21	180.8±11.4	160.7±10.4
3rd trimester	20	213.4±10.6	269.6±20.2
During delivery	18	165.9±13.0	345.9±30.8
Immediately after delivery	11	185.9±18.2	353.0±45.2
2 months after delivery	13	133.8±16.3	127.1±14.9
Toxaemia of pregnancy	5	Range: 170-260	440-744

Factor VIII activity and antigen in pregnancy, during delivery and in the puerperium. Mean and standard error of the mean.

Table VI

<u>Patients</u>	<u>Factor VIII</u>	
	<u>Activity</u>	<u>Antigen</u>
20	92.4±4.0	88.5±5.5

Factor VIII activity and antigen in 20 women using oral contraceptives. Mean and standard error of the mean.

The levels of factor VIII activity and antigen in term newborn infants are shown in Fig 2b, Paper 8. They are compared with those in fetuses with crown-heel lengths of 9-30 cm. The values were lower in fetuses than in term newborn infants ($p < 0.001$). Both activity and antigen were, however, readily demonstrated even in small fetuses. A slight but significant correlation ($p < 0.05$) was found between activity and antigen.

Discussion

The site of production and the distribution of factor VIII in tissues have been studied in several ways. Perfusion studies and transplantation experiments in animals have produced some evidence that the liver, and also the spleen, can produce and release factor VIII (Weaver et al 1964, Norman et al 1968, Dodds et al 1972, Webster et al 1971). However, several clinical workers (Zetterquist and v.Francken 1963, Meili and Straub 1970) found that factor VIII could be high even in severe liver disease. It is evident from the present investigation that the liver cannot be the only or probably not even the major site of synthesis. The high levels of activity are accompanied by high, or even higher levels of antigen, which proves that the high activity is not an artefact due to intermediates in the clotting process. This conclusion is further strengthened by chromatography of cryoprecipitate from a patient with acute total liver necrosis (Paper 7). Factor VIII protein was isolated in a much larger amount than from normal cryoprecipitate and on gel filtration on Sepharose 6 B it behaved like factor VIII from normal cryoprecipitate (Fig. 1, Paper 7). Furthermore, the subunit structure was the same (Fig. 2, Paper 7). The high plasma level in this patient cannot be due to a release from damaged liver cells, like the release of transaminases, as it continued to be high in the absence of any functioning liver parenchyma (low transaminases) for 2 months. Similar results have been obtained by Green and Ratnoff (1974) in a study of alcoholic cirrhosis of the liver.

Some evidence produced by other workers points to a multifocal synthesis of factor VIII in the organs of the RES, in reticuloendothelial cells and in cells concerned with the formation of blood cells (Niléhn 1962, Zacharski 1969, Ponn et al 1971). Tuddenham et al (1974) found synthesis of factor VIII antigen in cultured fetal liver and spleen. Bouhasin et al (1971) noted a remarkable rise in factor VIII activity in a patient with mild haemophilia who developed acute lymphoblastic leukaemia. Jaffe et al (1973) found some production of factor VIII antigen in ordinary (fetal, it is true) endothelial cells.

Wherever the site of synthesis may be, it is obvious that many diseases have a profound influence on factor VIII in plasma. The present investigation shows that conditions with an increased tissue breakdown and tissue repair are associated with a large amount of factor VIII in plasma. The increase seems to be quite unspecific, as it is encountered in many different disorders and it seems merely to

reflect the extent of tissue damage and repair. In pregnancy factor VIII increases with the growth of the uterus and the fetus. The further increase during actual delivery can be regarded as an acute reaction to stress, as that to infusion of adrenaline (Prentice et al 1972) and physical exercise (Bennett and Ratnoff 1972), probably with release of factor VIII from pools in the spleen for example. The excessive increase in toxæmia is probably due to increased tissue destruction. The hormonal influence of oral contraceptives does not affect factor VIII activity or antigen, at least not in normal women (but perhaps in von Willebrand's disease, see page 16).

Factor VIII activity and antigen levels in term newborn infants are not comparable with those in the mother. In the fetuses the values are lower than in the term infants, but even in the smallest ones both activity and antigen are detectable. These findings are important as they open up possibilities of prenatal diagnosis of haemophilia A and von Willebrand's disease (type I), if methods for sampling of uncoagulated fetal blood in vivo can be made available.

The discrepancy in many diseases between factor VIII activity and antigen calls for an explanation. It cannot be ascribed simply to methodological difficulties and a rapid decline in vitro of an excessively high activity in vivo. Green and Ratnoff (1974), like us, found an excess of antigen over activity in many cases of alcoholic cirrhosis of the liver. They also found that the antigens from cirrhotic and normal plasma, respectively, showed complete immunological identity and had the same electrophoretic mobility.

In the present series the antigen values generally tended to be higher than the activities. Factor VIII antigen is present in serum in the same amount as in plasma, although the protein has lost its procoagulant activity (Holmberg, unpublished). Even if there was no overt signs of intravascular coagulation in the cases presented in Paper 7, it is possible that there was an increased consumption of the clot promoting structures of the factor VIII molecule, *i.e.* in postoperative patients and in patients with severe burns. The opposite situation with an excess of activity over antigen was not often encountered. That such a clinical situation can exist is shown by the patient described in Paper 4. The ultimate answer to the question must abide clarification of the detailed structure of the factor VIII molecule.

III. NORMAL AND HAEMOPHILIC FACTOR VIII - BIOCHEMICAL CHARACTERISATION AND COMPARISON

Background

Methods for isolating factor VIII were not available until recently, for which reason the biochemical characterisation of the factor has only just begun. Various techniques for partial purification were the first steps towards complete separation. In 1956 Blombäck and Blombäck described a method for purifying fibrinogen by extracting Cohn's fraction I with citrate-buffer containing 6.5 % ethanol and 1 M glycine. This produced fraction I-0, which was stable and had a high factor VIII activity. Equally therapeutically important was the discovery by Pool et al (1964) that factor VIII could be cryoprecipitated when freshly frozen human plasma was slowly thawed in the cold. Wagner et al (1964) partially purified the protein by precipitating adsorbed plasma with glycine and β -alanine. Johnson and co-workers (Newman et al 1971) cryoprecipitated fresh frozen plasma with 3 % ethanol during thawing, adsorbed the extracted precipitate with $\text{Al}(\text{OH})_3$ and precipitated most of the fibrinogen with polyethylene glycol. Brinkhous et al (1968) obtained a high-potency concentrate by precipitating fibrinogen from cryoprecipitate and then precipitating factor VIII with glycine. But all these concentrates for therapeutic use are contaminated with other proteins.

Particularly separation of factor VIII from fibrinogen has proved difficult. Simonetti et al (1961) precipitated fibrinogen from fraction I-0 with tannic acid, and Green (1971) used a snake venom (Arvin). Ion exchange chromatography has been used for the same purpose (Michael and Tunnah 1963, Veder 1966).

A real advance was the introduction of material for gel filtration of large macromolecules. Hershgold et al (1967) and Johnson et al (1967) were the first to show that factor VIII was eluted together with very high molecular weight proteins. Preparations with a high degree of purity have been obtained by van Mourik and Mochtar (1970) and by Legaz et al (1973).

It has been shown (section I) that haemophilic plasma contains a protein that behaves like normal factor VIII on gel filtration on Sepharose 6 B and shows complete immunological identity with normal factor VIII. The aim of the work of this section was to isolate normal and haemophilic factor VIII and to compare them in respect of their primary chemical structure.

Results

The purification procedure was essentially according to van Mourik and Mochtar (1970), where cryoprecipitate is chromatographed on Sepharose 6 B, equilibrated with dextran (see Paper 9).

Factor VIII protein was isolated individually from several healthy volunteers and from 5 patients with haemophilia A. On SDS-polyacrylamide gel electrophoresis according to Weber and Osborn only one band was visible in both the normal and haemophilic preparations. They had the same mobility, and the estimated molecular weight of the chain was 180,000.

This protein chain was prepared from normal and haemophilic protein by reduction with dithiothreitol and alkylation with iodoacetic acid. The chain was cleaved in two ways: 1) with cyanogen bromide and 2) with trypsin. The cyanogen bromide fragments were compared with isoelectric focusing in polyacrylamide gel. The tryptic fragments were compared with finger-printing (high-voltage electrophoresis respectively chromatography). The pattern of cyanogen bromide fragments was very reproducible and no consistent difference between any of the haemophilic samples compared to the normals was detected. The finger-prints could not either disclose a difference in primary structure between normal and haemophilic factor VIII.

Discussion

The results of the present investigation suggest that factor VIII is composed of identical subunits, or at least of subunits of roughly equal size. These subunits may be held together partly by covalent bonds and partly by noncovalent bonds (see Paper 9).

The subunit of normal protein and that of haemophilic protein were compared in two ways. The isoelectric focusing technique is capable of detecting small differences in charge, at least in peptides with a molecular weight below 20,000. Yet no difference was found between controls and five haemophiliacs, probably representing five different mutations. Neither was any difference found in the pattern of tryptic peptides, but in some parts of the maps the resolution was unsatisfactory. It can only be concluded that the most elaborate techniques available were not able to demonstrate a difference in structure between normal and haemophilic factor VIII.

This lack of a demonstrable difference might be explained by the assumption that factor VIII activity does not reside in the large

protein itself and thus not in its subunit, but in a smaller molecule, loosely attached to the larger one. Data on the subunit structure of factor VIII are indeed conflicting. Like us, Marchesi et al (1972), Legaz et al (1973), Shapiro et al (1973) and Bennett et al (1973) found only one subunit in normal factor VIII protein; the latter two also in haemophilic protein. All these investigators used SDS-electrophoresis after reduction of disulfide bonds according to Weber and Osborn (1969). van Mourik et al (1974), however, succeeded in dissociating the protein by dialysing it against phosphate buffers of low ionic strength into two components with respectively fast and slow electrophoretic mobility and with different immunological properties. But neither of these components had factor VIII or vWf activity.

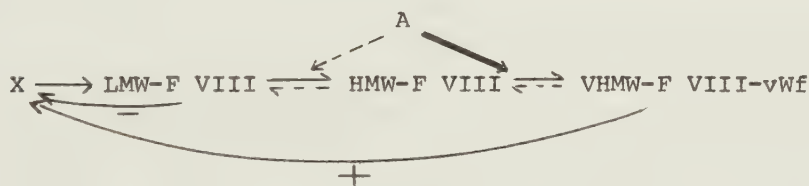
High ionic strength can also dissociate factor VIII protein. The first evidence of this was obtained by Thelin and Wagner (1961), who found that bovine factor VIII had a lower sedimentation rate in the ultracentrifuge at high ionic strength than at physiological ionic strength. This finding was confirmed by Weiss and Kochwa (1970) for human factor VIII. Two regions of factor VIII activity were found in human plasma, a fast sedimenting one and a slow sedimenting one. The factor VIII activity in cryoprecipitate was of the fast sedimenting type, while the activity remaining in the supernatant was of the slow sedimenting type.

Weiss et al (1972) and Owen and Wagner (1972) obtained similar results by gel filtration. Owen and Wagner dissociated canine factor VIII with 0.25 M CaCl_2 into a small molecular weight component with factor VIII activity and a large molecular weight component without activity ("carrier"). Reassociation of the components was possible when Ca was removed (Cooper et al 1973), but the low molecular weight component could not aggregate in the absence of the "carrier". The results of Austen (1974), however, on human material, suggest that such repolymerisation of small molecular weight factor VIII by itself to a high molecular weight factor VIII can occur. Rick and Hoyer (1973) demonstrated that the high and low molecular weight components of factor VIII formed by CaCl_2 -dissociation had common antigenic determinants detectable by antibody (both rabbit and human) neutralisation, but only the high molecular weight compound precipitated with the antibody. Weiss and Hoyer (1973) showed that this component retained Ristocetin cofactor activity even when it had lost its factor VIII procoagulant activity. Similar results with a separation of

factor VIII activity from a platelet aggregation factor in bovine antihaemophilic factor preparations were obtained by Griggs et al (1973).

The dissociation of factor VIII activity from factor VIII antigen and Ristocetin cofactor activity suggests that plasma factor VIII consists of two separate components united to form a complex, which has all the activities. But the demonstration of common antigenic determinants (Rick and Hoyer 1973) in the components suggests that the intact complex is a polymer that comprises repeating subunits. vWf activity (see section II) and Ristocetin cofactor activity appear to be found only in the polymeric forms, but factor VIII activity also in the monomeric or oligomeric forms. The present investigation supports the concept of repeating subunits.

How can the molecular defects in haemophilia A and vWd be explained from available experimental data? A tentative explanation is illustrated below:



From an X-chromosomal gene a protein chain with factor VIII activity is coded (LMW-F VIII = low molecular weight factor VIII). This chain polymerises to form a higher molecular weight compound (HMW-F VIII). Under the influence of, or together with the gene product(s) of, autosomal genes a very high molecular weight substance (VHMW-F VIII-vWf) is formed, which also has vWf activity (see section II) and precipitates visibly with a rabbit antibody. In haemophilia A and sex-linked vWd, type II, the VHMW-F VIII-vWf compound forms normally. The defect is in the primary structure of the chain coded from the X-chromosomal gene. In ordinary haemophilia A the primary haemostasis is normal and the VHMW-F VIII-vWf must therefore be normal regarding vWf-activity. In sex-linked vWd, type II, described in this work, it must be assumed that the X-chromosomally coded chain, even if it makes up only a portion of the VHMW-F VIII-vWf compound, affects the compound in such a way that its efficiency in primary haemostasis is decreased.

In the other types of vWd the autosomal gene products are defective, which leads to a reduced amount of VHMW-F VIII-vWf or an abnor-

mally polymerised product. Infusion of factor VIII concentrate (which consists of VHMW-F VIII-vWf) to a patient with classical vWd will briefly stimulate the production of LMW-F VIII, which, however, cannot be normally polymerised and incorporated in the VHMW-F VIII-vWf and thus the production of LMW-F VIII will soon cease. This mechanism can account for the results of the transfusion experiments (section I). Confirmation or refutation of this line of thought must abide further research in the ultrastructure of the factor VIII molecule.

SUMMARY

1. A factor VIII related protein is invariably demonstrable in plasma from patients with haemophilia A. This holds whether the haemophilia is mild, moderate or severe as well as in the presence of an anti-coagulant. The protein content is, if anything, somewhat higher than that of normal plasma. Normal and haemophilic factor VIII protein are composed of very similar subunits. The cyanogen bromide and tryptic fragments of the respective subunits are identical as far as can be judged with available techniques.

2. von Willebrand's disease is a heterogeneous condition. Several types of molecular defects were established. The following variants were distinguished in the present work:

a) von Willebrand's disease, type I, characterised by low factor VIII antigen and low factor VIII activity. Other characteristics are: prolonged bleeding time, decreased platelet adhesiveness (Salzman), reduced or no aggregation by Ristocetin, retarded increase in factor VIII activity after infusion of factor VIII concentrate. Autosomal dominant mode of inheritance.

b) Variant of von Willebrand's disease, type I, characterised by low factor VIII antigen and normal factor VIII activity. Other characteristics are: prolonged bleeding time, decreased platelet adhesiveness (Salzman), aggregation by Ristocetin only slightly sub-normal, retarded increase in factor VIII activity after infusion of factor VIII concentrate. Autosomal recessive mode of inheritance.

c) von Willebrand's disease, type II, X-chromosomal, characterised by normal factor VIII antigen, low factor VIII activity. Other characteristics are: prolonged bleeding time (Ivy), as a rule, decreased platelet adhesiveness (Salzman), aggregation by Ristocetin normal. No retarded increase in factor VIII activity after infusion of factor VIII concentrate. Probably X-chromosomal inheritance; the disease can therefore be regarded as a variant of haemophilia with clinical and laboratory resemblance to vWd.

d) von Willebrand's disease, type II, autosomal, characterised by normal factor VIII antigen, low factor VIII activity. Other characteristics are: prolonged bleeding time, decreased platelet adhesiveness (Salzman), reduced aggregation by Ristocetin, retarded increase in factor VIII activity after infusion of factor VIII concentrate. Reduced cryoprecipitability of factor VIII protein. Autosomal dominant mode of inheritance.

e) von Willebrand's disease, type II, characterised by normal factor VIII antigen and normal factor VIII activity. Other characteristics are: prolonged bleeding time, decreased platelet adhesiveness (Salzman), reduced aggregation by Ristocetin. Retarded increase in factor VIII activity after infusion of factor VIII concentrate. Mode of inheritance not demonstrated.

3. Factor VIII antigen normally occurs in vessel walls. The plasma content varies largely with the concentration of the antigen in the vessel walls. In severe von Willebrand's disease, type I, factor VIII antigen is missing in the vessel walls. Infusion tests suggest production of factor VIII antigen locally in the vessels.

4. Factor VIII antigen as well as factor VIII activity increases in various diseases, especially in those associated with extensive tissue damage and repair, and in pregnancy. The increase is not specific and reflects only the existence of a reactive process. Excess antigen over activity is common in reactive processes.

5. Factor VIII antigen as well as factor VIII activity is demonstrable in small fetuses. It might therefore prove possible to diagnose haemophilia A and severe von Willebrand's disease, type I prenatally.

Appendix 1 a

			Factor VIII		Bleed.time		Pl.adh.		R.aggr.	Symptoms
			Act.	Ant.	Ivy	Duke	Sz	B		Remarks
Fam. 1	III:5 (EK)		20	0	-	>30	10			Severe
	V:5 (BT)		8	0	-	>30	7			Severe
Fam. 3	III:1 (MH)		31	16	>30	-				Mild
Fam. 4	III:3 (AB)		44	20	10	4				Mild
Fam. 6	III:6 (AF)		60	36	19	5	3			Mild
	IV:2 (RF)		6	7	>30	>30	10			Severe
Fam. 8	II:1 (RB)		5	1	>30	>30				Severe
Fam. 9	III:1 (ES)		11	0	>30	-				Severe
Fam. 10	III:1 (MA)		10	<1	>30	>30	0	0	Absent	Severe
Fam. 11	II:1 (GB)		24	6	>30	>30	3	5	Reduced	Severe
Fam. 12	IV:2 (KPA)		5	0	>30	>30	2	10		Severe
Fam. 14	II:3 (BJ)		20	11	>30	-	0			Mild
	III:2 (SC)		31	48	22	3	38			Mild
	III:3 (BGJ)		19	8	>30	8	0			Mild
	III:4 (GJ)		28	6	>30	6-30	0			Mild
	III:5 (LJ)		38	19	>30	3-6	0			Mild
	IV:2 (SuC)		54	36	27					None
	IV:3 (RJ)		25	8	>25	3	10			Mild
Fam. 16	III:1 (AKP)		6	0	>30	>30				Severe
Fam. 17	IV:5 (LL)		41	20	>30	2	61			Mild
Fam. 22	II:1 (IDI)		47	40	>30					Mild-Sev
Fam. 23	III:12 (KW)		26	5	>30	>30	13			Severe
Fam. 30	III:2 (AW)		31	30	12					Mild
Fam. 32	III:5 (MJ)		49	28	19	5				Mild
	IV:1 (AJ)		9	0	>30	>30				Severe
Fam. 33	IV:1 (UR)		12	42	30	20				Mild
Fam. 38	III:5 (IS)		63	24	17	2				Mild
	IV:3 (JS)		42	6	11	3				Mild
	IV:4 (KPS)		30	8	>30					Mild
Fam. 40	II:8 (PW)		50	24		10				Mild
	III:3 (AMW)		10	0	>30	>30				Severe
Fam. 42	II:4 (HN)		43	18	>30	8	0			Mild
	II:5 (AS)		47	33	>30	2	0			Mild
	II:7 (IA)		40	39	>30	4	5			Mild
	II:9 (SP)		29	27	>30	24	2			Mild
	III:2 (IN)		48	25	30	2	35			Mild
	III:4 (AF)		36	16	>30	5	7			Mild
	III:27 (KZ)		50	35	30	-	0			Mild
	III:28 (KE)		29	23	>30	-				Mild
	III:32 (NA)		54	39	-	-	10			Mild
	III:33 (BA)		38	30	28	4	1			Mild
	III:35 (KS)		43	12	-	23	7			Mild
	III:39 (JIA)		44	27	>30	2	6			Mild
	III:40 (LA)		58	50	17	1	0			Mild
	III:42 (MP)		55	53	>30	5	39			Mild, Pregnant
	IV:7 (JEF)		48	17	15	2	31			Mild
	IV:33 (LH)		41	28	25	3				Mild
	IV:39 (JN)		-	17			0			?
	IV:44 (MZ)		45	30	30	4	18			Mild
	IV:45 (AZ)		35	25	24	-	3			Mild
	IV:46 (ME)		-	32	-	-				Mild
	IV:47 (HE)		30	12	-	4				Mild
	IV:61 (AA)		37	27	>30	3	19			Mild
	IV:62 (GA)		-	28			4			Mild
	IV:63 (OA)		33	19	19	5				Mild

		Factor VIII		Bleed.time		Pl.adh.		R.aggr.	Symptoms
		Act.	Ant.	Ivy	Duke	Sz.	B		Remarks
Fam. 42	IV:64 (AA)	60	23	16		8			Mild
	IV:65 (PS)	21	19	>25	8-15	34			Mild
	IV:66 (BIS)	53	23	>30	4	0			Mild
	IV:70 (AK)	44	20	>30	5				Mild
	IV:76 (FS)	35	9	12	3				Mild
	V:3 (CH)	44	27	>30	30				?
Fam. 45	III:1 (RO)	28	20		30				Mild
Fam. 58	II:2 (KF)	45	23	>30	8	11			Mild
	III:8 (IF)	45	13	>30	7				Mild
	III:9 (AF)	40	37	>30	5-30	0			Mild-Sev
	IV:1 (HE)	38	33	21	5			7	None
Fam. 60	II:13 (IJ)	35	15	>30	8				Mild
	II:14 (MB)	48	17	23	2	18			Mild
	III:1 (MJ)	63	25	30	6				Mild
	III:2 (LJ)	30	7	>30	30				Severe
	III:5 (AB)	63	33	15	5	3			Mild
	III:6 (JB)	33	21	21	-	14			Mild
Fam. 89	II:2 (HN)	75	66	25	2	6			Mild 85y
	IV:1 (MN)	80	42	>30	10	16			Mild
Fam. 92	II:2 (GM)	98	68	14	4	7			Mild
	III:1 (AM)	5	0	>30	>30				P-pill Severe
Fam.103	I:1 (VW)	145	246						Ca ventr
	II:3 (AW)	16	5	>30	>30	20			Severe
Fam.105	III:1 (IN)	68	51	18		9			P-pills
Fam.122	II:2 (WR)	53	15	>30	>30	61			Mild
Fam.130	II:1 (MU)	35	19	24	17				Mild
Fam.131	I:1 (GN)	30	20	>30	30	3			Mild
	II:1 (AN)	49	23	27	4				Mild
	II:2 (UN)	38	20	>30	>30	11			Mild-Sev
	II:3 (ACN)	88	35	>30					Stress
Fam.135	III:2 (JH)	6	0		>30		1	<5	Severe
Fam.139	(PK)	74	50	21		6			Pregnant
Fam.140	(LFB)	25	10	24		18			Mild
Fam.141	(BD)	58	50	10		17			Mild
Fam.142	I:2 (KM)	47	30						Mild
	II:2 (UD)	38	29	14					Mild
Fam.143	II:2 (HS)	53	32						Mild
	II:3 (HS)	43	29						Mild
	I:1 (HS)	31	15						Mild
	II:1 (ES)	31	15						Mild
Fam.144	(KJ)	53	30	17		12			Mild
Fam.145	I:2 (MF)	40	30	22	4	8			Mild
	II:1 (AF)	35	31	>30	7	13			Mild
	II:2 (BF)	65	45	20	5	25			Mild
Fam.147	(AA)	31	29	12	2	12			M-komp

Pl.adh. = platelet adhesiveness

Sz = Salzman's method

B = Bowie's method

R.aggr. = Platelet aggregation by Ristocetin

Appendix 1 b

			Factor VIII		Bleed.time		Pl.adh.		R.aggr.	Symptoms
			Act.	Ant.	Ivy	Duke	Sz	B		
Fam. 28	II:4 (AS)		37	210	30	3	22	20	Normal	Severe
	II:6 (UBK)		38	110	19	4	15			Mild
	III:3 (LH)		47	112	>30	5	7			Mild
	III:4 (RH)	27-37	75	24	3	47	40			Mild
	III:5 (IN)	26-45	122	17	4	19				Mild
	III:6 (AK)	62	132	9-15	4	6				Mild
	III:7 (MK)	53	100	10	2	7				Mild
	IV:5 (IH)?	71	174	10	2	38				None
	IV:6 (MH)?	84	138	10	2	40	27			None
Fam. 55	II:2 (MBP)	48	120	14	5	0				Mild
	II:3 (LF)	52	122	15	3	-				Mild
	III:2 (BAP)	48	172	18	3	79				Mild
	III:3 (BP)	43	104	19	4	0				Mild
	III:9 (GF)	48	200	30	3					Mild
	III:10 (RF)	33	116	18	4					Mild
Fam. 67	II:1 (AA)	49	94	9-29	4-9	48				Mild
Fam. 88	II:2 (IH)	40	137	11- >30						
Fam.100	II:2 (VÅ)	34	50	14	2					Mild
	II:4 (MS)	38	62	16	3	30	81			Mild
	II:9 (SS)	65	120	10	2					Mild
	II:11 (KN)	50	205	10	3					Mild
	III:7 (AS)	10	147	15-30	3	19	54	130%		Mild-Sev
	III:11 (RMS)	38	80	8	2					Mild
	III:16 (MLN)	33	113	3						Mild
	III:17 (JN)	15	123	12-30	10					Mild
	III:18 (PON)	12	120	18						Mild
Fam.107	II:2 (MB)	45	124	>30	7	0-11				Mild
Fam.137	(MBH)	39-52	76-100	8	1	0-1				Mild
Fam. 5	III:5 (PP)	48	79	30	9	9		25%		Mild
	IV:1 (WP)	57	60	21	16	6		34%		Mild
Fam. 75	II:1 (BT)	75	83	16-18	5-7	18		54%		Mild
Fam.138	I:2 (MS)	80	71	12	4	2-11				Mild
	II:2 (HS)	53	76	19	4	14				Mild
Fam.146	II:1 (YA)	65	63	>30	4	14				Mild

Pl.adh. = platelet adhesiveness

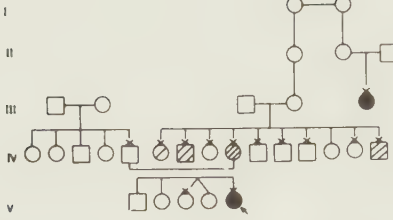
Sz = Salzman's method

B = Bowie's method

R.aggr. = Platelet aggregation by Ristocetin

Appendix 2a

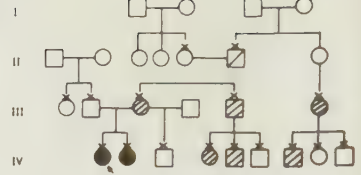
Family 1



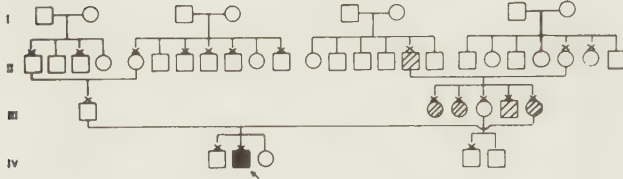
Family 3



Family 4



Family 5



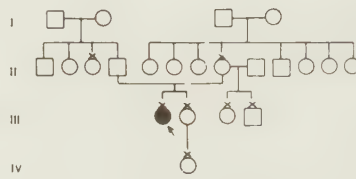
Family 8



Family 9



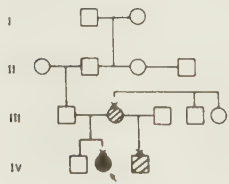
Family 10



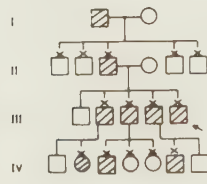
Family 11



Family 12



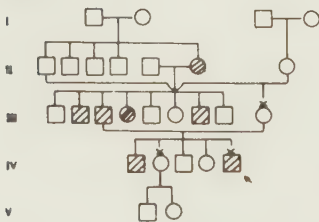
Family 14



Family 15



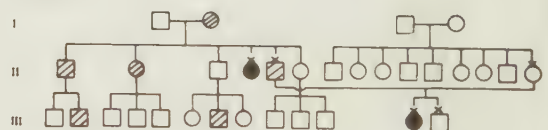
Family 17



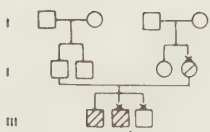
Family 22



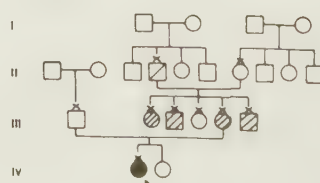
Family 23



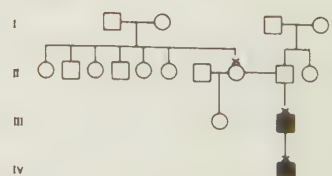
Family 30



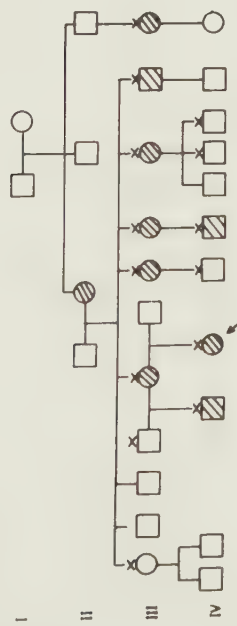
Family 32



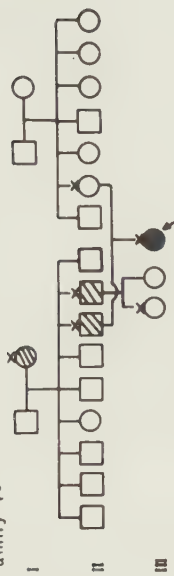
Family 33



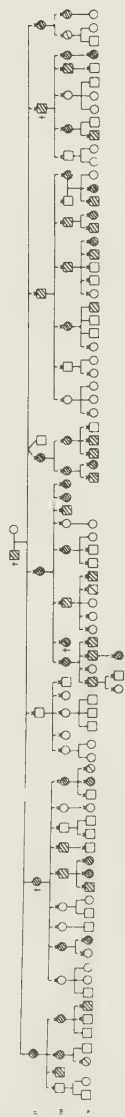
Family 38



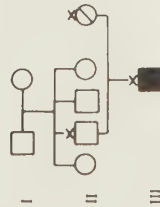
Family 40



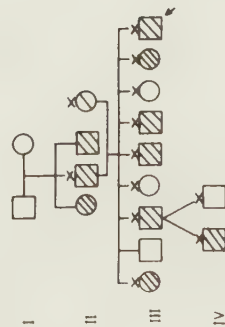
Family 42



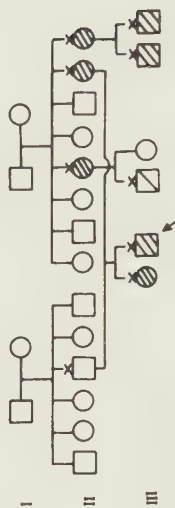
Family 45



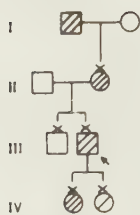
Family 58



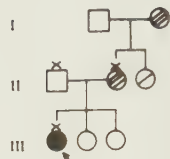
Family 60



Family 89



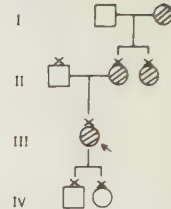
Family 92



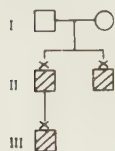
Family 103



Family 105



Family 122



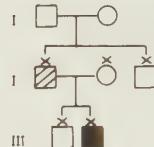
Family 130



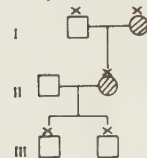
Family 131



Family 135



Family 142



Family 143

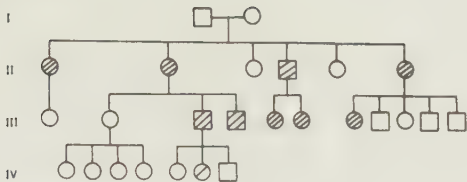


Family 145

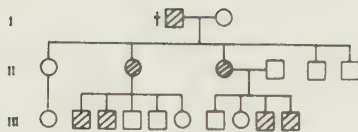


Appendix 2b

Family 28



Family 65



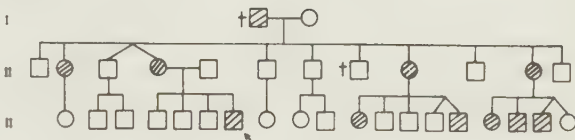
Family 67



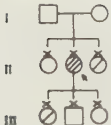
Family 88



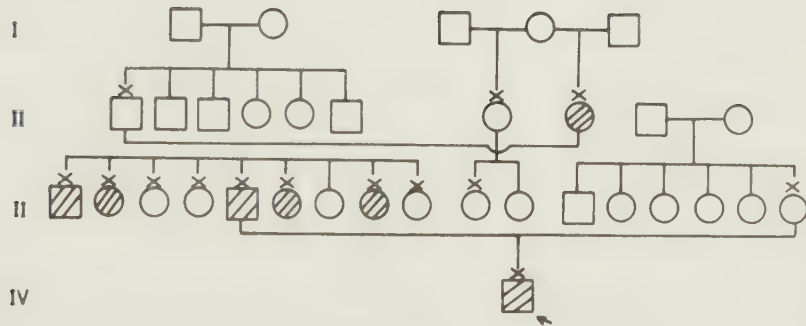
Family 100



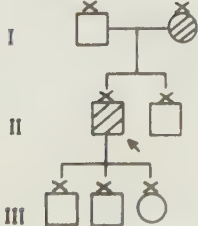
Family 107



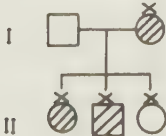
Family 5



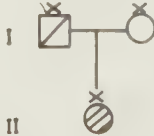
Family 75



Family 138



Family 146



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